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Effects of Gut Microbiota Manipulation by Antibiotics on Host Metabolism in Obese Humans: A Randomized Double-Blind Placebo-Controlled Trial

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SUMMARY

The gut microbiota has been implicated in obesity and cardiometabolic diseases, although evidence in humans is scarce. We investigated how gut microbiota manipulation by antibiotics (7-day administration of amoxicillin, vancomycin, or placebo) affects host metabolism in 57 obese, prediabetic men. Vancomycin, but not amoxicillin, decreased bacterial diversity and reduced *Firmicutes* involved in short-chain fatty acid and bile acid metabolism, concomitant with altered plasma and/or fecal metabolite concentrations. Adipose tissue gene expression of oxidative pathways was upregulated by antibiotics, whereas immune-related pathways were downregulated by vancomycin. Antibiotics did not affect tissue-specific insulin sensitivity, energy/substrate metabolism, postprandial hormones and metabolites, systemic inflammation, gut permeability, and adipocyte size. Importantly, energy harvest, adipocyte size, and whole-body insulin sensitivity were not altered at 8-week follow-up, despite a still considerably altered microbial composition, indicating that interference with adult microbiota by 7-day antibiotic treatment has no clinically relevant impact on metabolic health in obese humans.

INTRODUCTION

Accumulating evidence indicates that the composition of the gut microbiota plays a prominent role in body weight regulation and the development of type 2 diabetes mellitus (Greenhill, 2015; Khan et al., 2014). The gut microbiota regulates energy extraction from otherwise indigestible carbohydrates, determines the integrity of the intestinal epithelial layer, and influences the production and absorption of multiple signaling molecules involved in host metabolism. Several studies have demonstrated that germ-free mice are protected from diet-induced obesity, low-grade inflammation, and glucose intolerance as compared to conventionally raised animals (Bäckhed et al., 2004; Turnbaugh et al., 2006). Furthermore, it has been shown that transferring microbiota via fecal transplantation evoked alterations in body weight and insulin sensitivity in both rodents (Bäckhed et al., 2004) and humans (Hartstra et al., 2015; Vrieze et al., 2013) Taken together, these data indicate that modulation of the gut microbiota may provide a promising avenue to target obesity-related metabolic disorders (Cox and Blaser, 2013).

The gut microbiota composition can be modulated by, among others, prebiotics, probiotics, and antibiotics (Marchesi et al., 2015), thereby altering the presence and expression of microbial genes and derived metabolites such as bile acids (BAs) and short-chain fatty acids (SCFAs) (Canfora et al., 2015; Jones et al., 2014). Particularly, the use of antibiotics has been associated with increased metabolic impairments, mainly when exposure occurs in early life (Cox and Blaser, 2015; Jess, 2014). Of note, these findings are primarily based on animal studies, in

Table 1. Baseline Characteristics of the Study Population

	PLA (n = 19)	AMOX (n = 18)	VANCO (n = 19)
Age (years)	60.9 ± 1.7	55.7 ± 1.5	60.6 ± 1.5
Body weight (kg)	96.7 ± 2.3	96.3 ± 2.5	97.6 ± 1.9
Body mass index (kg/m^2)	31.0 ± 0.5	31.1 ± 0.8	31.5 ± 0.6
Waist/hip ratio	1.04 ± 0.01	1.04 ± 0.01	1.07 ± 0.01
Waist circumference (cm)	98.0 ± 8.1	101.1 ± 6.4	106.7 ± 6.3
Fasting glucose (mM)	6.0 ± 0.1	6.1 ± 0.1	6.1 ± 0.1
2 hr OGTT glucose (mM)	7.7 ± 0.4	7.0 ± 0.5	7.2 ± 0.4
Fasting insulin (mU/l)	15.7 ± 1.5	17.9 ± 1.6	16.8 ± 1.1
HOMA-IR	4.2 ± 0.4	4.9 ± 0.5	4.6 ± 0.3
HbA_{1c} (%)	5.5 ± 0.1	5.6 ± 0.1	5.6 ± 0.1

Data are mean ± SEM (n = 56). Homeostasis model assessment of insulin resistance (HOMA-IR), 75 g oral glucose tolerance test (OGTT), glycated haemoglobin (HbA_{1c}).

which the animals have mostly been exposed to a combination of antibiotics for periods varying from 2 to 20 weeks (Cani et al., 2008; Chou et al., 2008; Hwang et al., 2015; Membrez et al., 2008; Murphy et al., 2013). It has recently been shown that antibiotics may improve peripheral insulin sensitivity in a small number of obese subjects (Vrieze et al., 2014). Nevertheless, the effects observed in the latter study were relatively minor, and importantly, the study was not placebo-controlled. Thus, well-controlled, large human studies examining the effects and underlying mechanisms of microbiota modulation on host metabolism are currently lacking.

Here, we report on a randomized, double-blind, placebo-controlled trial that was designed to investigate the effects of broad- and narrow-spectrum antibiotic treatment for 7 days on gut microbiota composition, tissue-specific insulin sensitivity, energy expenditure, substrate oxidation, fecal and plasma BA and SCFA concentrations, gut permeability, abdominal subcutaneous adipocyte size, and systemic low-grade inflammation in obese men with impaired glucose homeostasis. Moreover, 8 weeks after cessation of antibiotic treatment, we again determined microbiota composition, whole-body insulin sensitivity (HOMA-IR), fecal energy harvest, and adipocyte size.

RESULTS AND DISCUSSION

Subject Characteristics

To study the role of the gut microbiota, we randomized 57 overweight and obese 35–70 year old Caucasian men to oral administration of the broad-spectrum antibiotic amoxicillin (AMOX), narrow-spectrum antibiotic vancomycin (VANCO, directed against Gram-positive bacteria), or placebo (PLA) for 7 days. No significant differences in baseline characteristics were present between groups (Table 1). All subjects had impaired fasting glucose levels (plasma glucose $\geq$ 5.6 mmol/l) and/or impaired glucose tolerance (2 hr plasma glucose during a 75 g oral glucose tolerance test 7.8–11.1 mmol/l) and were insulin resistant (homeostasis model assessment for insulin resistance; HOMA-IR > 2.2). One subject randomized to the AMOX intervention was considered a dropout due to use of other antibiotics during the study period. No serious adverse events and only a few cases of mild gastrointestinal discomfort were reported. There were no differences in daily energy and macronutrient intake, as monitored by a 3-day food diary, between and within groups before and after intervention. Furthermore, body weight remained unchanged for all treatment groups throughout the study period and at follow-up (data not shown).

Efficacy of Microbiota Manipulation by Antibiotic Treatment

The fecal microbiota composition was determined by analyzing 16S rRNA gene amplicons, using the Human Intestinal Tract Chip Microarray (HITchip) (Rajilić-Stojanović et al., 2009), which showed that 7-day VANCO markedly decreased microbial diversity ($p < 0.001$), whereas this was not affected by AMOX ($p = 0.42$) as compared to PLA (Figure 1). VANCO decreased the relative abundance of mainly Gram-positive bacteria of the *Firmicutes* phylum. Among the most strongly affected groups were genus-like groups that contain known butyrate-producing species from *Clostridium* clusters IV and XIVa, such as *Coprococcus eutactus*, *Faecalibacterium prausnitzii*, and *Anaerostipes caccae*, as well as species involved in BA dehydroxylation such as *Clostridium leptum*. Conversely, Gram-negative *Proteobacteria*, members of *Clostridium* cluster IX and VANCO-resistant Gram-positive *Bacilli* such as *Lactobacillus plantarum* and *Enterococcus*, showed increased relative abundance after VANCO treatment (Figure 1; Table S1), which is in line with previous studies (Vrieze et al., 2014; Yap et al., 2008). This pattern was confirmed with a supervised machine-learning technique (Random Forests analysis, Table S2). Importantly, microbiota composition was still affected 8 weeks after cessation of VANCO treatment. Microbial diversity was still lower ($q = 0.053$), and overall similarity and composition were deviant from baseline (pre-treatment) as compared to PLA. Although the bacterial groups that increased in abundance due to VANCO treatment had in general returned to baseline levels, several members of *Clostridium* clusters IV and XIVa were still decreased as compared to PLA. Furthermore, observed dynamics with respect to gut microbiota composition and diversity were individual specific (Figure S1). In contrast, AMOX treatment did not affect microbiota composition after 7 days treatment or at 8 weeks follow-up compared to PLA, which is in accordance with a previous study in obese humans (Vrieze et al., 2014).

Antibiotic Treatment Does Not Affect Tissue-Specific Insulin Sensitivity

The primary outcome of this study was peripheral insulin sensitivity (insulin-stimulated glucose rate of disappearance, Rd), as determined by a two-step hyperinsulinemic-euglycemic clamp with [6,6-$^2H_2$]-glucose tracer infusion. Antibiotic treatment did not significantly alter Rd as compared to PLA (Figure 2). Additionally, no effects were found on hepatic and adipose tissue (AT) insulin sensitivity, as determined by the insulin-mediated suppression of endogenous glucose production (EGP) and plasma free fatty acid (FFA) concentrations, respectively. In accordance, antibiotic treatment neither altered whole-body insulin sensitivity (HOMA-IR) immediately after cessation of treatment nor at 8 weeks follow-up (Figure S2). Our data are in contrast with several previous studies in rodents, which

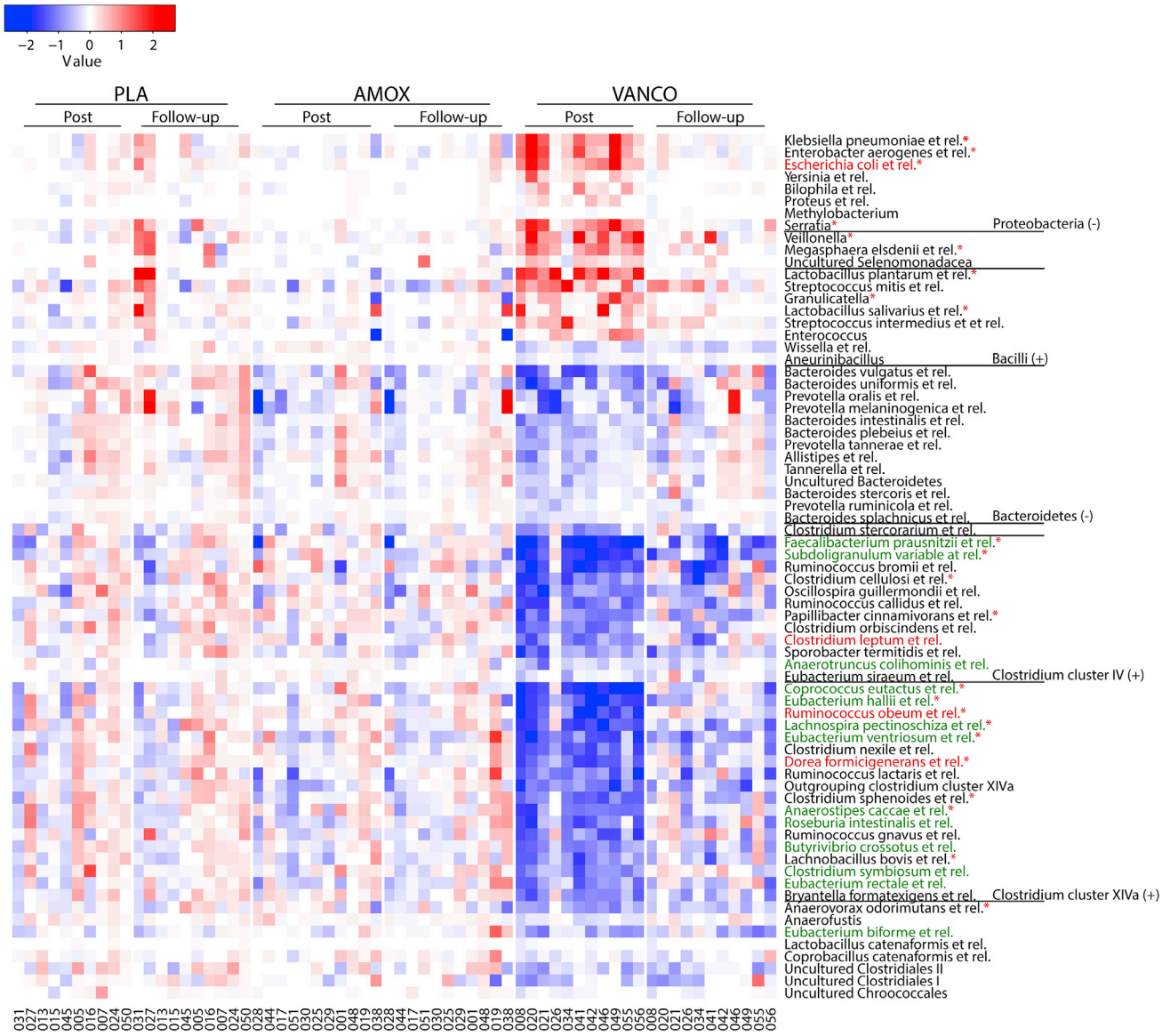

Figure 1. The Effect of Vancomycin and Amoxicillin Treatment on Microbiota Composition

Heatmap of bacterial groups (at genus and order like level with Gram staining between brackets) whose relative abundance was significantly different ($q < 0.05$) post-treatment within the VANCO group. Color value shows $\log_{10}$ fold changes compared to baseline. Genus like groups containing known butyrate producing and BA dehydroxylating species are depicted in green and red, respectively. * Groups that exhibited a significant difference between VANCO and PLA treatments. See also Figure S1 and Tables S1 and S2.

indicated that antibiotic treatment may improve glucose homeostasis and metabolic impairments (Bech-Nielsen et al., 2012; Carvalho et al., 2012; Chou et al., 2008; Hwang et al., 2015; Membrez et al., 2008; Murphy et al., 2013; Rune et al., 2013). Nevertheless, a more recent study showed that VANCO-treated mice had little weight change and no improvement in glycemic control (Rajpal et al., 2015). Consistent with the present data, a 4-day treatment with a broad-spectrum antibiotic cocktail did not affect postprandial glucose metabolism in lean, healthy men (Mikkelsen et al., 2015a). Furthermore, it has recently been shown in a limited number of obese subjects with the metabolic syndrome that VANCO slightly but significantly reduced peripheral insulin sensitivity, despite comparable changes in microbial composition and BA metabolism as found in the present study (Vrieze et al., 2014). Although the data of the latter study seems at odds with the present findings, it is important to emphasize that in the study by Vrieze and colleagues (Vrieze et al., 2014) the modest (~4%) VANCO-induced decrease in peripheral insulin sensitivity was based on a within-group comparison (post-treatment versus pre-treatment), since a placebo group was not included in the study design. Additionally, in the present study, follow-up measurements that were performed 8 weeks after treatment cessation also did not show an effect on whole-body insulin sensitivity, despite a still considerably

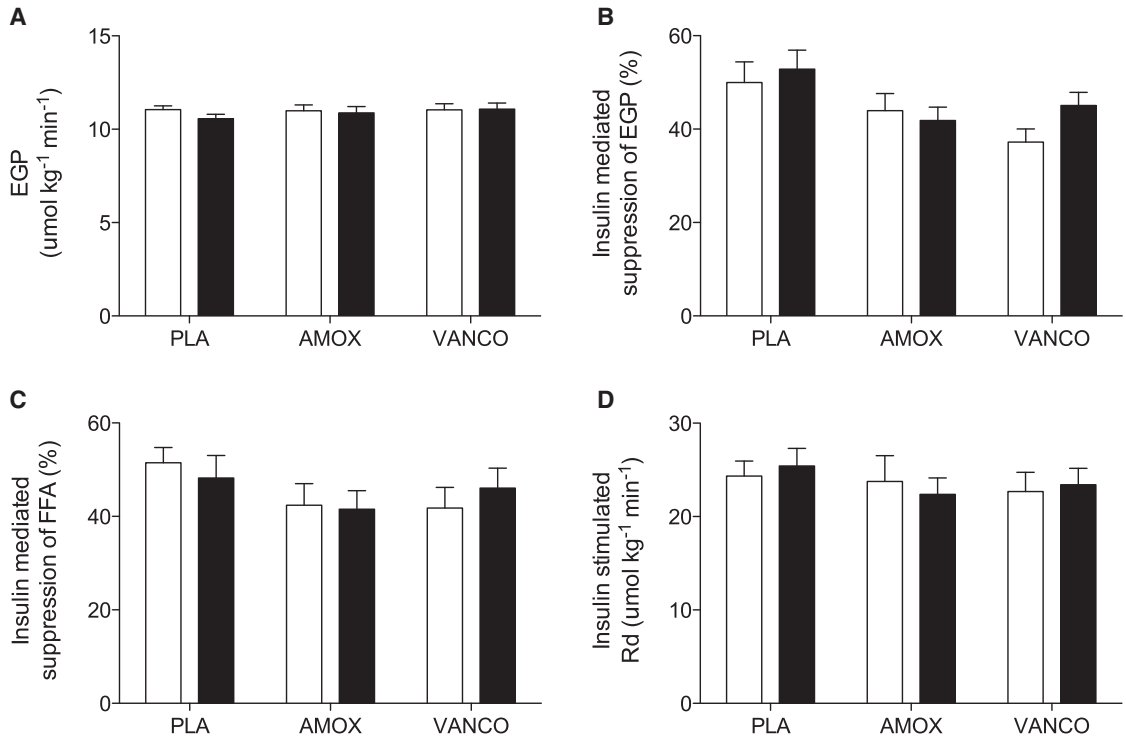

Figure 2. The Effect of 7 Days Placebo, Amoxicillin, and Vancomycin on Hepatic, Adipose Tissue, and Peripheral Insulin Sensitivity

Data are mean ± SEM (n = 56).

(A) (Fasting) liver endogenous glucose production (EGP).

(B) Steady-state insulin-mediated EGP suppression (%) upon 10 mU/m^2/min insulin infusion.

(C) Steady-state 10 mU/m^2/min insulin-mediated suppression (%) of circulating FFAs as measure for adipose tissue insulin sensitivity.

(D) 40 mU/m^2/min insulin-stimulated glucose disposal (Rd). See also Figures S2, S5, S6 and Table S4.

altered microbial composition as compared to pre-treatment as well as placebo.

Antibiotic Treatment Does Not Affect Energy and Substrate Metabolism

To examine the effect of gut microbiota modulation on post-prandial metabolite concentrations, energy expenditure, and substrate oxidation, we performed a high-fat mixed-meal test (2.6MJ [61E% fat, 33E% carbohydrates, 6E% protein]). We determined arterialized plasma metabolite concentrations and measured energy expenditure and substrate oxidation by whole-body indirect calorimetry. Neither VANCO nor AMOX significantly affected basal and postprandial plasma glucose, insulin, FFA, triacylglycerol (TAG), and lactate concentrations (Table 2; Figure S3; Table S3). Also, no significant effects on basal and postprandial energy expenditure, carbohydrate, and fat oxidation were found (Figure 3). After adjustment for fecal weight, intestinal energy harvest, which is reflected by daily fecal energy content, was neither changed immediately after treatment cessation, nor after 8 weeks follow-up (Figure 3). Although previous studies in rodents have shown a prominent role of the gut microbiota in energy harvest and body weight (Cani et al., 2008; Turnbaugh et al., 2006), our findings suggest that antibiotics do not alter energy harvest in humans. Of note, in rodent studies, animals were exposed to antibiotics in their drinking water for 2 up to 20 weeks (Bech-Nielsen et al., 2012; Cani et al., 2008; Carvalho et al., 2012; Chou et al., 2008; Hwang et al., 2015; Membrez et al., 2008; Murphy et al., 2013; Rune et al., 2013). Similarly, more prolonged treatment (4 to 6 weeks) with a higher dosage or a combination of different antibiotics increased body weight in endocarditis patients (Million et al., 2013; Thuny et al., 2010). These studies may indicate that a long-term dysbalance in microbiota composition has more pronounced effects as compared to short-term manipulation. However, it is hard to differentiate between the role of the gut microbiota and systemic effects of antibiotics in the latter studies. Noteworthy, we have applied a 2-day wash-out period before post-treatment measurements were performed to exclude that effects may be mediated via direct systemic effects of antibiotics. Additionally, VANCO does not pass the gastrointestinal barrier and, therefore, does not reach the circulation (Gonzales et al., 2010).

Antibiotic Treatment Does Not Alter Gut Permeability and Systemic Inflammatory Markers

We investigated the effect of 7 days of AMOX and VANCO treatment on gut permeability and the related translocation of bacterial lipopolysaccharide (LPS) from the intestinal lumen into the circulation. The pronounced VANCO-induced microbial alterations were not accompanied by changes in small intestine and proximal colon permeability (Figure S4), as assessed by a multi-saccharide test (van Wijck et al., 2013). This is in

Table 2. Effect of 7 Days Placebo, Amoxicillin, and Vancomycin on Metabolic, Inflammatory, and Hormonal Parameters

Fasting Plasma Metabolite, Hormone and ANGPTL4 Concentrations					
Variable		PLA (N = 14)	AMOX (N = 12)	VANCO (N = 12)	p value[a]
Glucose (mM)	Pre	6.31 ± 1.12	6.48 ± 0.25	6.25 ± 0.19	0.177[b]
	Post	6.29 ± 0.14	6.39 ± 0.20	5.99 ± 0.13	
TAG (μM)	Pre	1,404 ± 166	1,085 ± 151	1,027 ± 88	0.511
	Post	1,470 ± 215	1,034 ± 149	1,058 ± 101	
FFA (μM)	Pre	699 ± 34	683 ± 48	679 ± 38	0.423[b]
	Post	661 ± 34	579 ± 58	626 ± 54	
Lactate (mM)	Pre	0.80 ± 0.07	0.93 ± 0.04	0.88 ± 0.11	0.238
	Post	0.91 ± 0.11	0.90 ± 0.05	0.79 ± 0.06	
Insulin (mU/l)	Pre	11.5 ± 1.3	12.6 ± 1.3	14.3 ± 1.8	0.504
	Post	12.7 ± 1.6	13.4 ± 1.8	13.9 ± 1.5	
GLP-1 (pmol/l)	Pre	8.7 ± 0.7	8.5 ± 0.7	9.7 ± 1.1	0.670
	Post	9.3 ± 1.1	8.7 ± 0.8	10.2 ± 1.2	
Leptin (ng/ml)	Pre	11.4 ± 1.6	10.1 ± 2.1	9.7 ± 0.8	0.106[b]
	Post	12.9 ± 2.3	10.0 ± 1.8	8.8 ± 0.8	
ANGPTL4 (ng/ml)	Pre	5.1 ± 0.7	4.3 ± 0.5	4.9 ± 0.5	0.137
	Post	5.5 ± 0.7	3.8 ± 0.5	4.3 ± 0.3	
Fasting inflammatory marker concentrations					
		PLA (N = 19)	AMOX (N = 18)	VANCO (N = 19)	p value[a]
LBP (pg/ml)	Pre	19.6 ± 1.8	17.5 ± 1.8	25.7 ± 4.3	0.456
	Post	18.4 ± 3.3	20.4 ± 2.9	23.6 ± 3.6	
IL-6 (pg/ml)	Pre	0.8 ± 0.1	0.8 ± 0.1	1.0 ± 0.1	0.775
	Post	1.0 ± 0.1	0.8 ± 0.1	1.1 ± 0.1	
IL-8 (pg/ml)	Pre	6.2 ± 0.5	4.3 ± 0.4[c]	5.2 ± 0.4	0.444
	Post	5.9 ± 0.5	4.8 ± 0.4	5.9 ± 0.4	
TNF-α (pg/ml)	Pre	2.6 ± 0.1	2.3 ± 0.1[c]	2.7 ± 0.1	0.424
	Post	2.7 ± 0.1	2.5 ± 0.1	2.8 ± 0.1	

Data are mean ± SEM. For determination of plasma hormones and metabolites, only a subgroup of n = 38 was analyzed. There were no significant differences between the groups after intervention (Post) compared to baseline (Pre). Triacylglycerol (TAG), free fatty acids (FFA), glucagon-like peptide (GLP), angiopoietin-like 4 (ANGPTL4), lipopolysaccharide-binding protein (LBP), interleukin (IL), tumor necrosis factor (TNF).

[a]p value represents the overall intervention effect between groups assessed by repeated-measures ANOVA (time × treat p value) or ANCOVA when baseline concentrations were different between groups.

[b]Time effect ($p < 0.05$).

[c]Baseline group difference ($p < 0.05$). See also Figures S3 and S4 and Table S3.

accordance with unchanged LPS-binding protein (LBP) concentrations after VANCO and AMOX treatment as compared to PLA (Table 2). LPS, which is released by Gram-negative bacteria, may trigger the immune system by increasing inflammatory cytokine production in AT and is frequently used as an indicator of metabolic endotoxemia (Cani et al., 2007). Therefore, we have additionally determined plasma interleukin (IL)-6, IL-8, and tumor necrosis factor (TNF)-α concentrations. In line with unchanged LBP concentrations, neither of these inflammatory factors was affected by 7-day VANCO or AMOX as compared to PLA. This was observed despite a substantial increase in relative abundance of potentially pro-inflammatory Gram-negative *Proteobacteria.*

Vancomycin Inhibits BA Conversion and SCFA Production

SCFAs, notably butyrate, can be produced by several groups within the *Firmicutes* phylum (mainly *Clostridium* clusters XIVa and IV, including *Coprococcus eutactus* and *F. prausnitzii*), some of which are also involved in BA dehydroxylation (Jones et al., 2014; Ridlon et al., 2006). Indeed, we found a decreased relative abundance of these groups after VANCO, which was accompanied by a marked reduction in plasma ($p = 0.005$) and fecal ($p = 0.001$) concentrations of secondary BAs as compared to PLA (Figure 4). This was accompanied by an increase of fecal primary BAs ($p = 0.013$). In addition, fecal SCFA concentrations (acetate ($p = 0.001$), butyrate ($p < 0.001$), caproate ($p < 0.001$), and valerate ($p = 0.009$)) were significantly decreased following VANCO, while in plasma only butyrate tended to decrease after VANCO ($p = 0.078$) but not following AMOX treatment (Figure 5).

Although BAs and SCFAs may control incretin release (Brighton et al., 2015; Canfora et al., 2015) and affect energy metabolism in rodents (Gao et al., 2009), no effects on postprandial energy and substrate metabolism and fasting and postprandial glucagon-like peptide 1 (GLP-1) concentrations were found in the present study (Tables 2 and S3).

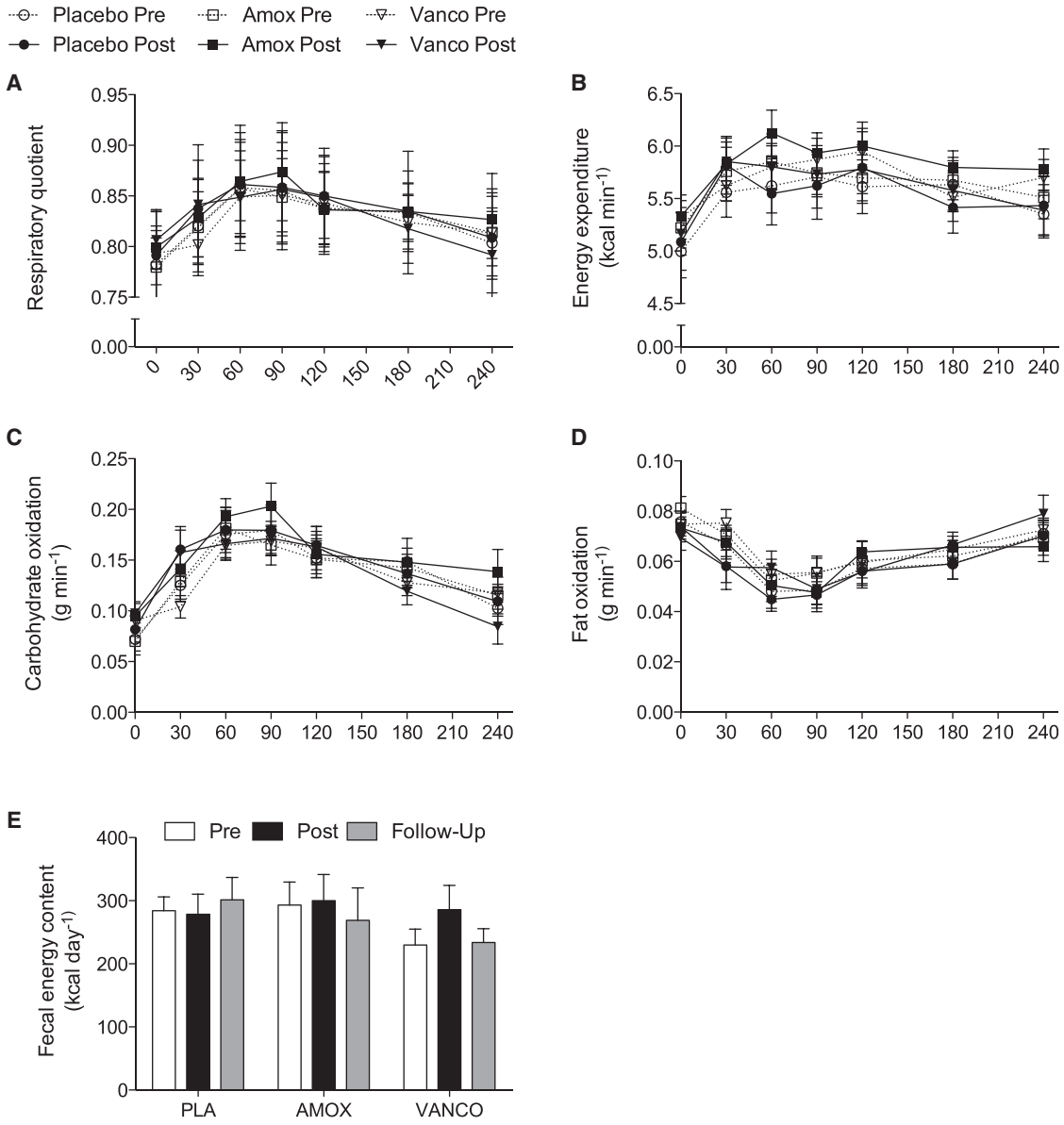

Figure 3. The Effect of 7 Days Placebo, Amoxicillin, and Vancomycin on energy Expenditure, Substrate Metabolism, and Fecal Energy Excretion

Data are mean ± SEM. Indirect calorimetry was performed during fasting conditions and for 4 hr after intake of a liquid high-fat mixed meal (HFMM) in a subgroup of n = 37. Mean O_2 consumption and CO_2-production over 20 min were used for calculations.

(A–D) Fasting respiratory quotient (RQ), energy expenditure (EE), carbohydrate oxidation, and fat oxidation did not differ after intervention (time × treat p value > 0.05). Incremental AUCs after ingestion of HFMM were also not affected by AMOX or VANCO.

(E) Fecal energy excretion (kcal/day) did not significantly change after VANCO or AMOX compared to PLA (n = 56).

Antibiotic Treatment Alters Adipose Tissue Gene Expression but Not Adipocyte Morphology

To determine the effect of an altered gut microbiota composition on AT, we collected abdominal subcutaneous AT biopsies to examine adipocyte size and gene expression profiles using Affymetrix microarray transcriptomic analysis. Antibiotic treatment had no significant effect on abdominal subcutaneous adipocyte size and the proportion of small and large adipocytes, neither directly after treatment cessation nor at 8 weeks follow-up (Figure S5). Remarkably, when comparing the gene expression data with the Kyoto Encyclopedia of Genes and Genomes (KEGG), we found that VANCO and, to a lesser extent, AMOX increased AT expression of genes involved in pathways related to peroxisome-proliferator activated receptor (PPAR)-signaling and of genes encoding proteins involved in the mitochondrial Krebs cycle, fatty acid degradation, and other components of the oxidative machinery, suggestive of increased oxidative metabolism in AT (Figure S6). In addition, VANCO decreased the expression of

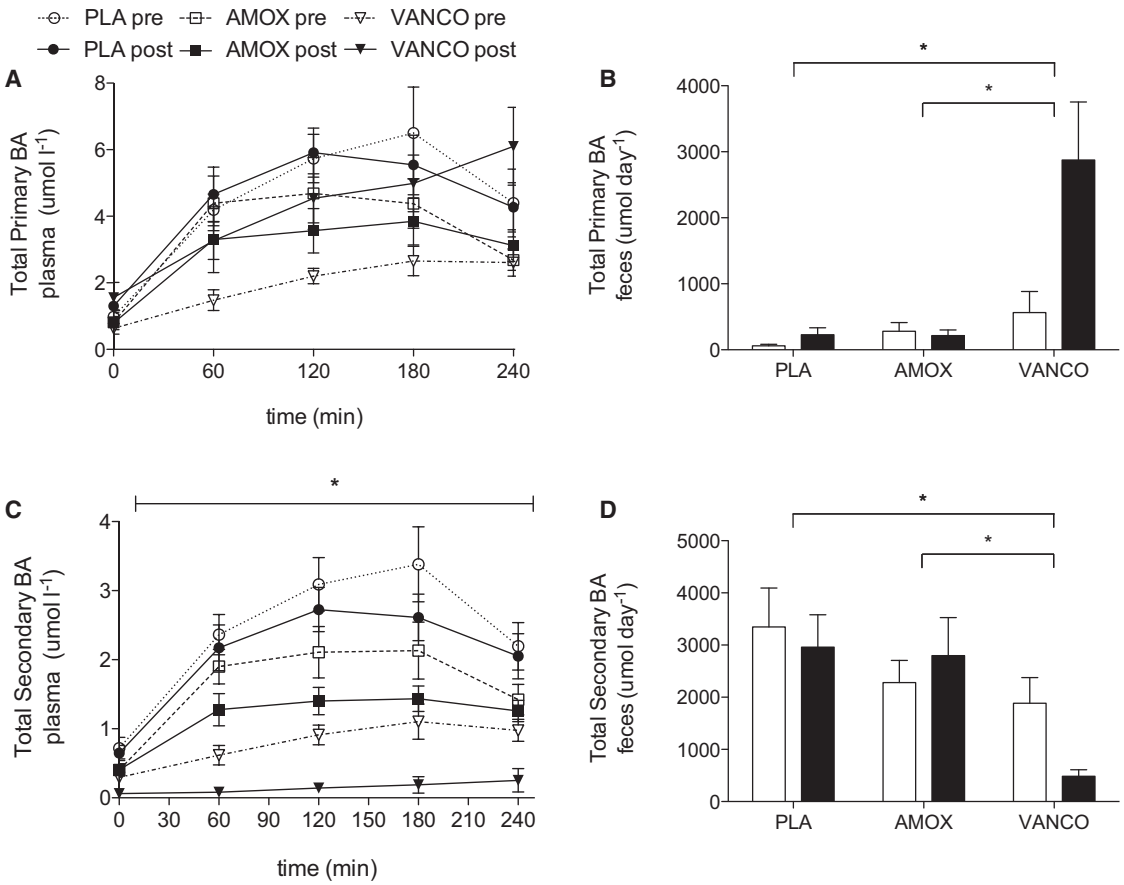

Figure 4. The Effect of 7 Days Placebo, Amoxicillin, and Vancomycin on BA Concentrations in Plasma and Feces

Data are mean ± SEM. In a subgroup of n = 37.

(A) Plasma postprandial primary BA did not change significantly.

(B–D) VANCO increased fecal primary BA (B), decreased plasma secondary BA (C), and fecal secondary BA (D) compared to PLA and AMOX. * time × treat p value < 0.05 for VANCO versus PLA.

histone clustering genes. Although we found no differences in adipocyte morphology and circulating FFA, TAG, leptin, and angiopoietin-like 4 (ANGPTL4) concentrations (Table 2), these alterations in the AT transcriptome may translate into changes in AT function over longer periods of time.

Finally, VANCO decreased the expression of gene sets involved in apoptosis and nuclear factor NFκB signaling as well as adaptive and innate immune responses, including genes of major histocompatibility complex-I, T cell, B cell, and natural killer cell signaling. In contrast, genes related to lysosomal breakdown were upregulated as compared to PLA (Table S4). Lower NFκB-dependent gene expression and diminished NK and $CD8^+$ T cell function in macrophages have been observed in germ-free and antibiotic-treated mice (Ganal et al., 2012). In the latter study, the effects were ascribed to a reduced activation of Farnesoid X receptors by a reduction of unconjugated and secondary BAs (Jones et al., 2014), which seems in line with the present findings. In addition, although the exact role of SCFAs in the systemic and AT immune cell responses is unknown, SCFAs may be involved in the regulation of T cells in the gut and peripheral tissues via the G protein-coupled receptor 43 (Brestoff and Artis, 2013; Canfora et al., 2015; Fukuda et al., 2011; Furusawa et al., 2013). Despite the effects of antibiotic treatment on the KEGG pathways described above, no significant associations (FDR < 0.25) were found between individual bacterial groups and AT gene expression (data not shown).

Microbial Groups Are Not Associated with Host Metabolic Parameters

Although overall host metabolism did not change significantly following antibiotic treatment, we used univariate and multivariate statistics (redundancy analysis) to assess possible associations between specific characteristics of gut microbial profiles and host metabolic parameters. However, we did not find any significant and consistent associations when we evaluated the abundance and dynamics of individual bacterial taxa, combinations of taxa, the complete microbiota, and bacterial diversity at baseline, as well as 7 days and 8 weeks post-intervention.

Furthermore, we investigated whether we could identify and connect patterns of specific metabolic and/or microbiological perturbations with the response to the intervention. First, we evaluated the stratification of subjects based on the extent of the microbial shift in diversity, as well as microbial composition. Second, based on the extent and direction of the metabolic

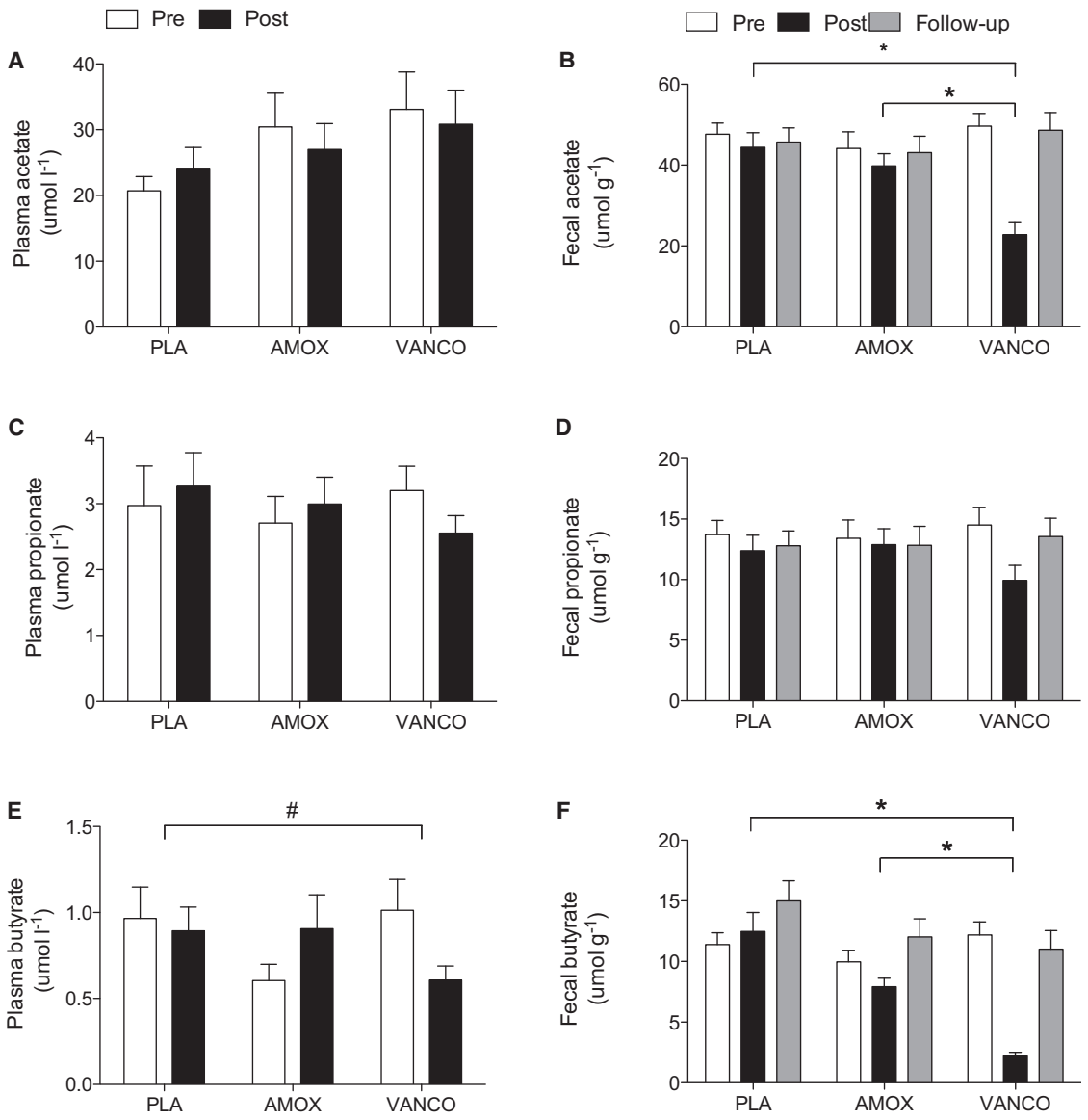

Figure 5. The Effect of 7 Days Placebo, Amoxicillin, and Vancomycin on Plasma and Fecal SCFA Concentrations

(A and C–E) Data are mean ± SEM (n = 56). No significant effect was found for (A) plasma acetate, (C) plasma propionate, (D) fecal propionate, and a trend (#p = 0.07) for (E) plasma butyrate.

(B and F) Fecal acetate and butyrate decreased after VANCO treatment but not after AMOX. * time × treat p value < 0.05 for VANCO versus PLA and versus AMOX.

response to the intervention, we used univariate and cluster analysis to discover microbial patterns. Lastly, we used latent class analysis (McCutcheon, 1987) to define groups of subjects with certain metabolic patterns before and after treatment. Neither of these analyses showed groups of individuals with specific associations of the microbiota with host metabolic parameters (data not shown).

PERSPECTIVES

In the present study, we demonstrated that seven days VANCO treatment markedly affected microbial diversity and composition, which was accompanied by a reduced conversion of primary to secondary BAs and a lower production of SCFAs in the gut. Importantly, these alterations did not translate into significant effects on peripheral, hepatic and AT insulin sensitivity, energy and substrate metabolism, and systemic low-grade inflammation immediately after treatment cessation. Moreover, no clinically relevant effects on energy harvest, abdominal subcutaneous adipocyte size, and whole-body insulin sensitivity (HOMA-IR) were found at 8 weeks follow-up. In contrast to VANCO, no effects of AMOX treatment on gut microbial composition, and metabolic and inflammatory parameters were found. Taken together, the present study implies that interference with a

resilient adult microbiota by antibiotics has no clinically relevant short-term (7 days) and long-term (8 weeks) effects on the metabolic parameters measured in this study. This contradicts many previous rodents studies and again highlights that rodent data cannot always be extrapolated to humans.

Noteworthy, several nuances have to be made with respect to the conclusions of the present study. First, since we studied obese, insulin-resistant men with impaired glucose metabolism, we cannot exclude that microbiota manipulation by antibiotics may have more pronounced effects in women or less metabolically compromised individuals. Second, the duration of the intervention was relatively short, compared to rodent studies. Furthermore, it has been demonstrated that the risk of developing type 2 diabetes was increased when subjects were exposed to >5 antibiotic treatments (Mikkelsen et al., 2015b) and that the number of prescriptions may accelerate the aging-related decline of intestinal integrity (Kerr et al., 2015). Of note, the participants that were included in the present study had received on average 1.7 antibiotic treatments over the past 10 years, without any antibiotic use 3 months prior to the start of the study. As mentioned above, several studies have indicated that a long-term or more frequent perturbation in microbiota composition may have more pronounced effects on metabolic health than short-term manipulation. For this reason, it is important to emphasize that the present study does not exclude an important role for the gut microbiota manipulations in changes of host metabolism. This should be further investigated in future prospective and long-term (dietary, prebiotic, and/or probiotic) intervention studies in humans.

EXPERIMENTAL PROCEDURES

Study Participants

57 low-active (<3 hr organized sports activities per week), weight-stable (<2 kg body weight change 3 months prior to inclusion) overweight/obese (BMI 25–35 kg/m^2) Caucasian men, between 35 and 70 years with impaired glucose metabolism (either fasting glucose >5.6 mmol/l, and/or 2 hr glucose between 7.8–11 mmol/l) and HOMA-IR > 2.2 were included in this study (https://ClinicalTrials.gov, NCT02241421). Subjects were recruited via advertisements in local newspapers and were all living in the area around Maastricht, The Netherlands. All subjects gave written informed consent for participation in this study, which was reviewed and approved by the local Medical Ethical Committee of Maastricht University Medical Center+. All procedures were according to the declaration of Helsinki (revised version, October 2008). Exclusion criteria were the use of antibiotics for a period of 3 months before entering the study, known allergic reactions to any type of antibiotics; hearing disorders; cancer; liver malfunction; major illnesses with a life expectancy less than 5 years and pulmonary; hepatic, cardiovascular, kidney, and gastrointestinal disease. Subjects did not use β-blockers, lipid- and glucose-lowering drugs, anti-oxidants, or chronic corticosteroids.

Study Design and Randomization

This randomized, placebo-controlled, double-blind study had a three-armed parallel design. Participants were randomized to oral intake of amoxicillin (broad-spectrum antibiotic), vancomycin (directed against Gram-positive bacteria), or placebo (microcrystalline cellulose) for 7 consecutive days (1,500 mg/day). Antibiotics and placebo were equally capsulated to blind the content to subjects and investigators (BasicPharma, The Netherlands). The allocation sequence was established by computer-generated randomization (https://nl.tenalea.net). Block-randomization with stratification for BMI, age, and 2 hr-glucose values was used to increase the homogeneity of the treatment arms (block size, n = 6). After completion of the study, returned capsules were counted to assess compliance. Participants were asked to maintain their habitual physical activity pattern and dietary habits (monitored by 3-day food diaries) throughout the study. The evening before an investigation day, a low-fiber, low-fat meal was consumed. Before and after intervention, study measurements were conducted following a 10-hr overnight fast. To ensure complete systemic and gastrointestinal clearance of antibiotics, a 2-day wash-out period was taken into account before post-treatment measurements. Participants returned for a follow-up visit 8 weeks after treatment cessation.

Hyperinsulinemic-Euglycemic Clamp

A two-step hyperinsulinemic–euglycemic clamp combined with a [6,6-$^2H_2$]-glucose tracer (Cambridge Isotope Laboratorieswas performed to measure Rd, EGP (Hulzebos et al., 2001) and the insulin-mediated suppression of FFAs (DeFronzo et al., 1979; Steele, 1959). Blood samples were taken from a superficial dorsal hand vein, which was arterialized by using a hot-box (~50°C). After a bolus-injection (2.4 mg/kg), tracer-infusion was started at 0.04 mg/kg/min, which was continued throughout the measurement. After 2 hr, low-dose insulin was infused at 10 mU/m^2/min for 2 hr (Kotronen et al., 2008), followed by high-dose insulin at 40 mU/m^2/min for 2 hr (Brehm et al., 2006). By variable co-infusion of a 17.5% glucose solution, enriched by 1.1% tracer, plasma glucose concentrations were maintained at 5.0 mmol/l. For calculation of steady-state kinetics, additional blood samples were taken in the last 30 min of each step (0, 10, and 40 mU/m^2/min insulin).

Postprandial Test

Blood was sampled from a superficial dorsal hand vein, which was arterialized by placing the hand into a hot-box (~50°C). Blood samples were taken during the fasting state (t-30, t-15, t0 min) and postprandial (t = 30, 60, 90, 120, 180, and 240 min) after ingestion of the test meal. The liquid test meal, that was consumed within 5 min, provided 2.6 MJ (61 E% fat, 33 E% carbohydrate, 6 E% protein), which was consumed within 5 min at t = 0 (Most et al., 2016).

Indirect Calorimetry

For indirect calorimetry during fasting (30 min) and the 4-hr postprandial state, the open-circuit ventilated hood system was used (Omnical, Maastricht University) (Schoffelen et al., 1997). Calculations of energy expenditure and substrate oxidation were performed according to the formulas of Weir (1949) and Frayn (1983). Nitrogen excretion was based on the assumption that protein oxidation represents ~15% of total energy expenditure (Jans et al., 2012).

Gut Permeability Test

After baseline urine collection, subjects drank a 150 ml multisaccharide test mix [1 g sucrose (Van Gilse, Dinteloord, the Netherlands), 1 g lactulose (Centrafarm, Etten-Leur, the Netherlands), 1 g sucralose (Brenntag, Sittard, the Netherlands), 1 g erythritol (Danisco Sweeteners, Copenhagen, Denmark), and 0.5 g of l-rhamnose (Danisco)] (van Wijck et al., 2013). Urine was collected for determination of the urinary sucrose concentration in the 0–120 min urine collection, representing gastro-duodenal permeability, whereas in this collection small intestinal permeability is represented by the lactulose/rhamnose ratio. Proximal colon permeability is represented by the suclarose/erythritol ratio of the 120–300 min urine collection.

Biochemical Analyses for Plasma Variables

Blood was collected into pre-chilled tubes and centrifuged at 1,000 × *g*, and plasma was snap-frozen and stored at −80°C until analyses. Isotopic enrichment of plasma glucose was determined by electron ionization gas chromatography-mass spectrometry and expressed as tracer-to-tracee ratio for steady-state calculations of Rd and EGP (Hulzebos et al., 2001). Plasma glucose, lactate, FFA, and glycerol were determined with the Cobas Fara auto-analyzer (Roche, Switzerland). Plasma insulin was measured with a double antibody radioimmunoassay (Millipore). Plasma leptin concentrations were analyzed using commercially available radioimmunoassay kits (Human Leptin RIA, Millipore Corporation). Plasma ANGPTL4 concentrations were measured by ELISA as described (Kersten et al., 2009). Plasma concentrations of IL-6, IL-8, and TNF-α were determined using a multiplex enzyme-linked immunosorbent assay (Human ProInflammatory II 4-Plex Ultra-Sensitive Kit, Meso Scale Diagnositics). Isocratic ion-exchange HPLC (Model PU-1980 pump) with mass spectrometry (Model LTQ XL, Thermo Fisher Scientific) was used

to determine sugar concentrations in plasma and urine for gastrointestinal permeability assessment (van Wijck et al., 2013). LBP was measured using non-commercial ELISA as described before (Schols et al., 1996). Plasma concentrations of GLP-1 were measured by radioimmunoassays as previously described (Vilsbøll et al., 2003). Plasma BA profile was measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Vrieze et al., 2014). The total amount of primary (cholic acid and chenodeoxycholic acid and their taurine and glycine conjugated forms) and secondary BAs (deoxycholic acid, lithocholic acid and their conjugated forms) was calculated as the sum of the individually quantified BA. Plasma SCFAs were determined by LC-MS/MS as reported before (van Eijk et al., 2009). The detection limits for acetate, propionate, and butyrate were 0.1, 0.05, and 0.05 μmol/l, respectively.

Laboratory Analysis of Adipose Tissue

Abdominal subcutaneous AT biopsies were taken under local anesthesia under fasted conditions. One portion was embedded in paraffin. Sections were cut for staining, digital imaging, and computerized morphometric measurement of individual adipocytes (Goossens et al., 2011). One portion (~500 mg) was snapfrozen in liquid nitrogen, from which RNA was extracted (Trizol chloroform extraction, Invitrogen) and used for microarray analysis. 100 ng total RNA was labeled by Whole-Transcript Sense Target Assay and hybridized to human whole-genome Affymetrix Gene 1.1 ST arrays, targeting 19,793 unique genes (Affymetrix). Quality control and data analysis pipeline have been described in detail previously (Lin et al., 2011). Individual genes on the array were defined as changed when comparison of the normalized signal intensities showed a FDRq < 0.05 in a two-tailed paired t test with Bayesan correction (Limma) (Smyth, 2004). Further functional data analysis was performed on the filtered dataset with Gene Set Enrichment Analysis (GSEA, http://www.broad.mit.edu/gsea). Gene sets were selected based upon FDRq < 0.2.

Laboratory Analysis of Feces

Feces was collected at home for 2 consecutive days at baseline and 7 days and 8 weeks after intervention using the BMP commode specimen collection system, and divided over sterile tubes at home. Subjects were provided with a box of dry ice to freeze their stool samples immediately after defecation at approximately −80°C and for transport to the university. Total fecal amount was weighed, and 24-hr fecal samples were used to determine energy content using adiabatic bomb calorimetry (CBB 330, standard benzoic acid 6,320 cal/g, BCS-CRMno90N). 24-hr fecal BA composition was determined by using gas chromatography (GC) as described before (Hulzebos et al., 2001). Fecal SCFAs were measured by gas chromatography-mass spectrometry (GC-MS, Medical laboratory 'Dr. Stein & Collegae' Germany), according to the method described before (García-Villalba et al., 2012).

For microbiota profiling, DNA was isolated from 24-hr fecal samples as described before (Vrieze et al., 2014) and subsequently used for phylogenetic profiling using the HITchip phylogenetic microarray (Rajilić-Stojanović et al., 2009). Standardized quality control was maintained through our library of a duplicated set of 3,631 probes targeting the 16S rRNA gene sequences of over 1,000 intestinal bacterial phylotypes. A more detailed description of microbiota profiling procedures can be found in the Supplemental Experimental Procedures.

Statistics

The calculated sample size (n = 19 per treatment arm) was based on a 20% physiologically relevant change of insulin sensitivity (α = 0.05, β = 0.8). All data were evaluated for normality. Univariate analysis (ANOVA) was applied to compare group characteristics at baseline. Differences between treatments were analyzed using repeated-measures ANOVA with time and treatment as factors. ANCOVA analysis of the delta (post-pre value) was used for parameters when significantly different at baseline, taking the baseline value into account as covariate. The postprandial response (energy expenditure, substrate oxidation, and GLP-1) is given as incremental area under the curve (iAUC/min), which was calculated by the trapezoid method. For HITchip analysis, log10-transformed signals were used as a proxy for bacterial logarithmic abundance. To determine which bacterial groups were significantly different in relative abundance before and after treatment within each group, a paired Wilcoxon test was used. Between-treatment group effects were assessed with linear mixed models using the lme4 package (Bates et al., 2015). Benjamini-Hochberg correction was applied for multiple testing. We used Random Forests, a supervised machine-learning technique, and the pre- and post-treatment classes to confirm these results (Liaw and Wiener, 2002). To determine whether individuals could be grouped into classes of specific metabolic responses to the interventions, we used the lcmm R package (Proust-Lima et al., 2015) to perform Latent Class Analysis. Diversity of the microbiota was quantified based on non-logarithmized HITchip oligo-level signals by inverse Simpson's index using the Vegan package (Oksanen et al., 2011). ANOVA with Tukey's Honest Significant post hoc analysis was applied to compare diversity between and within groups. Data are expressed as means ± SEM, with a two-sided significance level of $p < 0.05$. Statistical analysis was performed using SPSS 20.0 for Macintosh and R 3.03.

ACCESSION NUMBERS

Array data have been submitted to GEO: GSE76003.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, four tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2016.06.016.

AUTHOR CONTRIBUTIONS

D.R., G.H.G., C.H.C.D., and E.E.B. designed the study and analyzed the data; D.R., G.H.G., and E.N. contributed to data acquisition; C.M.v.d.B. and J.M. were responsible for biopsies; K.L. contributed to gut permeability analysis; M.V.B. was involved in the microarray analysis; G.D.A.H., H.S., and E.G.Z. were involved in the HITchip analysis and reporting; A.K.G. contributed to combustion calorimetry and BA analysis; S.W.M.O.D. had primary responsibility for plasma SCFA analysis; J.J.H. contributed to GLP-1 analysis; M.N. and R.S.K. contributed to glucose-tracer analysis; D.R. wrote the manuscript; and E.E.B. had the primary responsibility for the final content. All authors revised the content of the manuscript and read and approved the manuscript for publication.

CONFLICTS OF INTEREST

The authors have declared that no competing interests exist.

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